J. Biochem. 128, 121–128 (2000)

# Structure-Specific Effects of Thyroxine Analogs on Human Liver 3α-Hydroxysteroid Dehydrogenase[1]

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Received March 15, 2000, accepted May 2, 2000

**The NADP(H)-linked oxidoreductase activity of a major isozyme of human liver 3α-hydroxysteroid dehydrogenase was activated 5-, 4-, and 2-fold by D-thyroxine ($T_4$), L-$T_4$ and DL-3,3′,5′-triiodothyronine (reverse $T_3$), respectively. Kinetic analysis of the activation indicated that D-$T_4$, L-$T_4$, and reverse $T_3$ are non-essential activators, showing binding constants of 1.5, 1.1, and 3.6 μM, respectively. Comparison of the effects of the $T_4$ analogs on the activities of the mutant enzymes suggests that the binding site is composed of at least Lys-270, Arg-276, and the C-terminal loop of the enzyme. L-$T_3$, DL-thyronine, and D-tyrosine had no effect on the enzyme, but 3,5,3′,5′-tetra- and 3,5,3′-tri-iodothyropropionic acids were potent competitive inhibitors with $K_i$ values of 42 and 60 nM, respectively, with respect to the substrate. The inhibition constant was lowered upon the activation of the enzyme by D-$T_4$, and the inhibition by the deamino derivatives of $T_4$ and $T_3$ disappeared upon modification of the C-terminal loop of the enzyme, but not upon replacement of Lys-270 or Arg-276 with Met. These results indicate that, depending on their structures, the $T_4$ analogs bind differently to two distinct sites at the active center of the enzyme to produce stimulatory and inhibitory effects.**

**Key words: activation, aldo-keto reductase family, binding site, 3α-hydroxysteroid dehydrogenase, thyroxine, triiodothyronine.**

3α-Hydroxysteroid dehydrogenase (3α-HSD, EC 1.1.1.213) is distributed in various tissues, and has recently been shown to be involved in the metabolism of nonsteroidal compounds, in addition to the biosynthesis and inactivation of steroid molecules (*1*, *2*). For example, 3α-HSD from rat and human liver exhibits prostaglandin oxidoreductase, carbonyl reductase and dihydrodiol dehydrogenase activities, and has been thought to play roles in the metabolism of prostaglandins, drug ketones and polycyclic aromatic hydrocarbons (*2–5*). Although one 3α-HSD species has been purified from rat liver, three multiple forms (DD1, DD2, and DD4) of 3α-HSD with dihydrodiol dehydrogenase activity have been isolated from human liver cytosol (*6*), and at least four types of cDNAs for the enzyme have been cloned (*7–11*). The human 3α-HSD isozymes are composed of 323 amino acids with more than 83% sequence identity, and belong to the aldo-keto reductase (AKR) superfamily. They are named AKR1C1–1C4 (*12*). AKR1C1, AKR1C2, and AKR1C4, and are identical to DD1, DD2, and DD4, respectively, and show distinct specificities for hydroxysteroids (*3*, *6*, *10*, *13*, *14*). DD1 exhibits higher activity for 20α-hydroxysteroids than for 3α-hydroxysteroids, DD2 oxidizes some 3α-hydroxysteroids, and DD4 shows broad and high 3α-HSD activity for various steroids. Recombinant AKR1C3 shows 17 β-HSD activity and low 3α-HSD activity for some steroids (*11*, *15*). The catalytic efficiency ($V_{max}/K_m$ value) of DD4 for most 3α-hydroxysteroids is higher than those of the other isozymes, and previous purification of the human liver 3α-HSD isozymes indicated that the predominant isozyme is DD4 (*3*).

An outstanding feature of DD4 is its activation by several drugs, including sulfobromophthalein (*16*), clofibric acid derivatives (*17*) and anti-inflammatory 2-arylpropionic acids (*18*). The structural requisites for the activating drugs have been shown to be the negatively charged sulfonyl or carboxyl group and hydrophobic aromatic ring(s). The negatively charged group has been suggested to interact with Lys-270 and/or Arg-276 of DD4, which are involved in the binding of the 2′-phosphate group of NADP(H) (*19*). Although the other components in the activator-binding site have not been determined, these findings suggest the presence of endogenous activators, which structurally mimic the drugs, and regulate the activity of this isozyme. In this study, we tested various biomolecules as activators of this isozyme, and found that thyroxine ($T_4$) and its analogs exhibit stimulatory or inhibitory effects on the enzyme depending on their structures: $T_4$ and 3,3′,5′-triiodothyronine ($rT_3$) activate the enzyme, whereas derivatives without an α-amino group on the side chain of $T_4$ and 3,5,3′-triiodothyronine ($T_3$) act as potent inhibitors. We examined the kinetic mechanism of the activation and inhibition by $T_4$ and

[1] This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan

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Abbreviations: AKR, aldo-keto reductase; HSD, hydroxysteroid dehydrogenase; $rT_3$, reverse $T_3$ (3,3′,5′-triiodothyronine), $T_4PA$, 3,5,3′,5′-tetraiodothyropropionic acid; $T_4$, thyroxine; $T_3$, 3,5,3′-triiodothyronine; $T_3PA$, 3,5,3′-triiodothyropropionic acid.

its analogs and their binding sites, using DD4 mutants and a chimeric enzyme in which the C-terminal 39 residues are replaced by those of DD1, which is not influenced by the analogs.

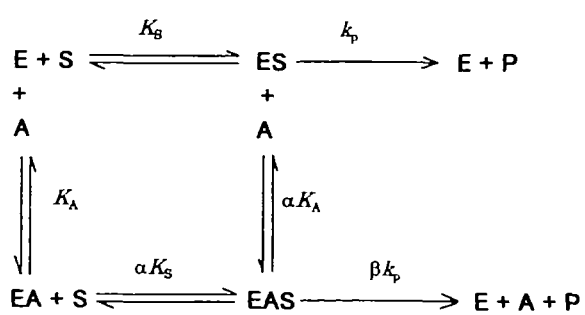
## MATERIALS AND METHODS

*Chemicals and Enzymes*—$T_4$, its analogs, steroids, ibuprofen and fenoprofen were purchased from Sigma Chemicals (St. Louis, MO). *S*-(+)-Indan-1-ol was obtained from Fluka Chemie AB (Buchs, Switzerland); $NADP^+$ was from Oriental Yeast (Tokyo); and sulfobromophthalein was from Nacalai Tesque (Kyoto) Other chemicals were obtained from Tokyo Kasei Organic Chemicals (Tokyo) and Aldrich (Milwaukee, WI). Recombinant DD1 (*13*), DD2 (*11*), DD4 (*14*), AKR1C3 (*11*), mutant enzymes (K270M and R276M) of DD4 (*19*) and the chimeric enzyme (CDD4-1) (*20*) were expressed in *Escherichia coli* and purified to homogeneity.

*Enzyme Assay*—Dehydrogenase activities of 3α-HSD isozymes were assayed fluorometrically or spectrophotometrically by recording the production of NADPH, as described (*16*). The standard assay for activity was performed in 2.0 ml of 0.1 M potassium phosphate, pH 7.4, containing 0.25 mM $NADP^+$, 2 mM *S*-indan-1-ol and enzyme The activities of the K270M and R276M mutant enzymes were determined with 0 5 and 5 mM $NADP^+$, respectively, in the above reaction mixture because of their high $K_m$ values for the coenzyme (*19*). The reductase activity was assayed with 0.1 mM NADPH and carbonyl substrate, instead of $NADP^+$ and *S*-indan-1-ol. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol NADPH/min at 25°C.

$T_4$ and its analogs were dissolved in methanol, and 50 μl was added to the reaction mixture before the reaction was started by the addition of the enzyme. Other carboxylic acids were dissolved in 10 mM NaOH and then neutralized with 10 mM HCl to form the sodium salts. The pH dependency of the enzyme activity was determined with 0.1 M potassium phosphate buffers (pH 6.5–10.0) prepared by mixing solutions of $H_3PO_4$ and $K_3PO_4$.

The $K_m$ values for the substrates were determined by Lineweaver-Burk analysis in the presence of five different substrate concentrations with a saturating $NADP^+$ concentration of 0.25 mM. The initial velocity analysis for determining the binding constant ($K_A$), α and β values for a non-essential activator (Scheme 1) was carried out with its six different concentrations with $NADP^+$ and *S*-indan-1-ol as the varied and fixed substrates, respectively, in the absence




Scheme 1 **Scheme for non-essential activation.** Abbreviations E, enzyme; S, substrate, P, product, A, activator

(control) and presence of different concentrations of the activator. The slope, intercept, $K_m$ and $V_{max}$ values of the Lineweaver-Burk plots were first calculated by a computer program for least-squares linear regression, and the $K_A$, α and β values were calculated from the secondary reciprocal plots of 1/Δ *slope* and 1/Δ *intercept versus* 1/[activator] (*21*). The Δ *slope* and Δ *intercept* values were obtained as the respective control values minus "plus activator" values from the individual Lineweaver-Burk plots. The constants α and β refer to the -fold change in $K_m$ and $V_{max}$, respectively, obtained in the presence of the activator. In the 1/Δ *slope versus* 1/[activator] replot, the *x* and *y* intercepts correspond to $-\beta/\alpha K_A$ and $\beta V_{max}/K_S$ (β–α), respectively, and the *y* intercept in the 1/Δ *intercept versus* 1/[activator] replot gives $\beta V_{max}/(\beta-1)$ The kinetic study in the presence of an inhibitor was performed in a similar manner, and the inhibition constant was determined by assuming the appropriate kinetic model for inhibition as described previously (*22*). All kinetic measurements were performed in triplicate, and the mean values were used for the subsequent calculation. All standard errors of fit were less than 15%, unless otherwise noted.

*Fluorescence Study*—Fluorescence measurements were obtained with a Hitachi F-2000 spectrofluorometer equipped with a temperature-regulated cell compartment. The effects of $T_4$, DL-r$T_3$, and L-$T_3$ on the intrinsic fluorescence (excitation wavelength 280 nm; emission wavelength 330 nm) of DD4 were measured at 25°C in 1.0 ml of 0.1 M potassium phosphate buffer, pH 7 4, by adding 0.5-μl aliquots of solutions of $T_4$ and its analogs. The observed relative fluorescence intensity was corrected for dilution of the protein. The binding data of the titration were calculated as previously reported by Okabe and Hokaze (*23*), and graphed as Scatchard plots using the following equation:

$$\frac{[L]_b}{[L]_f[E]_t} = -\frac{1}{K_d}\frac{[L]_b}{[E]_t} + \frac{n}{K_d}$$

where $[L]_b$ and $[L]_f$ are the bound and free concentrations of ligand, respectively, $[E]_t$ is the total enzyme concentration, $K_d$ is the dissociation constant of the enzyme-ligand complex, and *n* is the number of ligands bound to the enzyme.

## RESULTS AND DISCUSSION

*Effects of $T_4$ and Its Analogs on DD4*—Previous studies on the structural characteristics of drug activators for DD4 have suggested the presence of a negatively charged sulfonyl or carboxyl group and hydrophobic aromatic ring(s) (*16–18*). To find an endogenous activator for this enzyme, we examined the effects of haematin, bilirubin, fatty acids, amino acids and tyrosine metabolites on the $NADP^+$-linked *S*-indan-1-ol dehydrogenase activity. Amino acids (1 mM), fatty acids with a carbon chain length shorter than eight (0.1 mM) and phenylpyruvic acid (1 mM) had no influence on the enzyme activity, while haematin, bilirubin and fatty acids with a long carbon chain length (C12:0 – C20:4) inhibited about half of the enzyme activity at concentrations of 5–25 μM. A stimulatory effect was observed when *R*-phenyllactic acid, DL-4-hydroxyphenyllactic acid, D-$T_4$, and L-$T_4$ were added to the reaction mixture. *R*-Phenyllactic acid and DL-4-hydroxyphenyllactic acid activated the enzyme activity by up to 2-fold at high concentrations of 1 and 5 mM, respectively (data not shown), whereas D-$T_4$ and L-$T_4$ more effectively stimulated the enzyme activity (Fig. 1).

Although the effects of D-$T_4$ and L-$T_4$ at concentrations above 10 μM could be not determined because of their low solubility, they activated the enzyme activity in a dose-dependent manner. It should be noted that similar stimulatory effects of D-$T_4$ and L-$T_4$ were observed with 5 μM androsterone as the substrate. Since D-$T_4$ showed a slightly greater stimulation than L-$T_4$, the effects of several derivatives of $T_4$ (Fig. 2) were examined to determine the structurally important parts of the activating $T_4$. DD4 was also activated by racemic $rT_3$ (maximally 2.3-fold), but not by L-$T_3$, DL-thyronine or D-tyrosine, which indicates the structural importance of the iodo group at position 5′ for the activation by $T_4$. On the other hand, deamino derivatives of $T_4$ and $T_3$, 3,5,3′,5′-tetraiodothyropropionic acid ($T_4$PA) and 3,5,3′-triiodothyropropionic acid ($T_3$PA), showed significant inhibitory effects on the enzyme activity, indicating that the presence of an α-amino group on the side chain is also required for the stimulatory effects of $T_4$.

When the effects of pH on the activation of the *S*-indan-1-ol and androsterone dehydrogenase activities by D-$T_4$, L-$T_4$, and $rT_3$ were examined, their optimal stimulatory effects were observed at pH around 7.5 – 8.0, and no activation was observed below pH 6.5 (data not shown). Thus, the stimulatory effects of the $T_4$ analogs are pronounced in a physiological pH range, regardless of the structures of the substrates. This is similar to the pH dependency of activation by known drug activators (*16–18*), in which the deprotonated sulfonyl or carboxyl group of the drugs is thought to bind to the enzyme. The carboxyl and amino groups of L-$T_4$ are negatively and positively charged, respectively, at the optimal pH of activation, because the respective p$K_a$ values are 2.2 and 10 1. In addition to a protonated amino group, a negatively charged carboxyl group may be one of the structural requisites for activating $T_4$ analogs.

The activation efficiencies (maximum stimulation percentage/concentration required for half maximum stimulation, %/μM) for D-$T_4$ and L-$T_4$ are 130 and 170, respectively, which are much higher than those for clofibric acid derivatives (3–17, *17*) and anti-inflammatory 2-arylpropionic acids (1–13, *18*), and comparable to that for sulfobromophthalein (200, *16*) the best drug activator for DD4. Although the activation efficiency differs among the above activating compounds, $T_4$ and its analogs, similar to drug activators, did not activate the enzyme activities of other 3α-HSD isozymes, DD1, DD2, and AKR1C3. This suggests that $T_4$ and drug activators bind to an identical site on DD4. In addition, $T_4$PA, a potent inhibitor of DD4, did not inhibit the other 3α-HSD isozymes, which suggests that the DD4 isozyme possesses binding site(s) for stimulatory and inhibitory compounds.

*Activation Mechanism and the Binding Site of $T_4$ and $rT_3$*—The effects of D-$T_4$, L-$T_4$, and $rT_3$ on the kinetic constants of DD4 for several representative substrates were compared in both the forward and reverse directions at a fixed concentration of 1 or 5 μM (Table I). These compounds led to increases in both the $K_m$ and $k_{cat}$ values for most substrates, including steroids, compared with those in the absence of the activator. The extents of the increases in the two kinetic values differed depending on the substrates, and the $K_m$ values for lithocholic acid and pyridine-4-aldehyde decreased by the addition of activators. The difference in the changes in the kinetic constants among substrates might result from a conformational change in the substrate-binding site of the enzyme caused by activator binding. The $k_{cat}/K_m$ values for all the substrates also rose upon activation, indicating that the catalytic efficiency of DD4

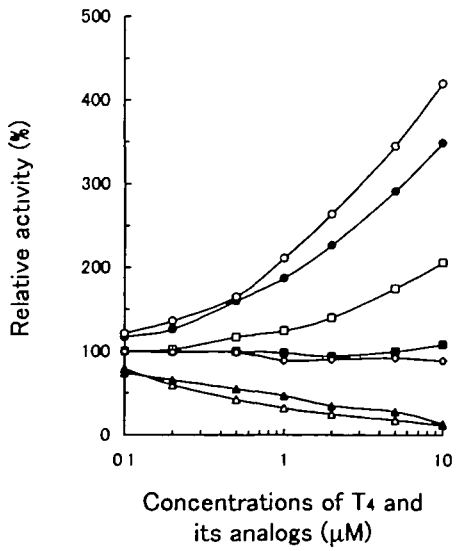



Fig. 1 **Effects of $T_4$ and its analogs on the *S*-indan-1-ol dehydrogenase activity of human liver DD4.** The activity was assayed in the presence of D-$T_4$ (○), L-$T_4$ (●), DL-$rT_3$ (□), L-$T_3$ (■), DL-thyronine (◇), $T_3$PA (▲), or $T_4$PA (△), and is expressed as the percent of control activity assayed in the absence of $T_4$ analogs.

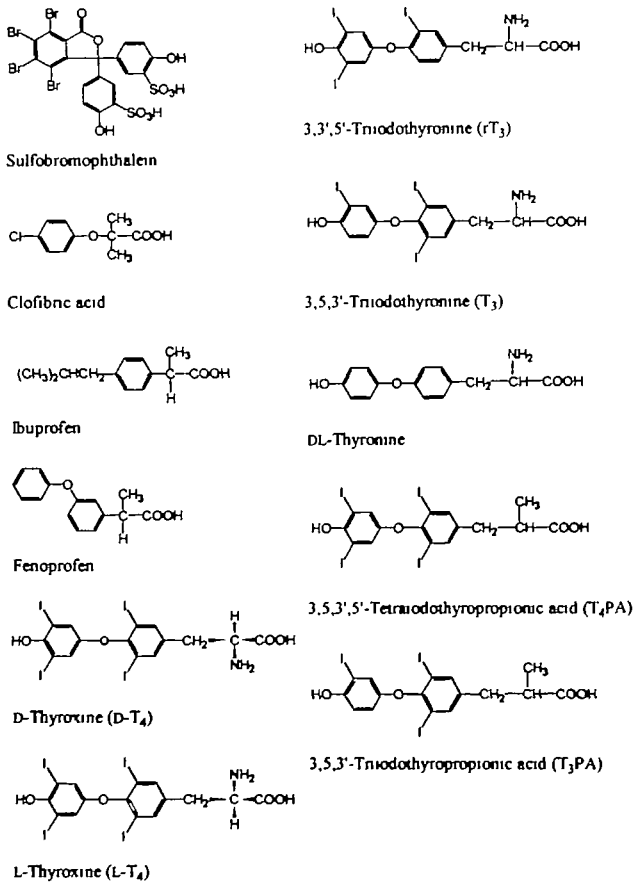



Fig. 2 **Structures of the activating drugs, $T_4$ and its analogs.**

TABLE I **Effects of L-$T_4$, D-$T_4$ and $rT_3$ on $K_m$ and $k_{cat}$ values for substrates.**

| Substrate | Without activator | | | 1 μM L-$T_4$ | | | 1 μM D-$T_4$ | | | 5 μM DL-rT | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $K_m$ (μM) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ | $K_m$ (μM) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ | $K_m$ (μM) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ | $K_m$ (μM) | $k_{cat}$ ($min^{-1}$) | $k_{cat}/K_m$ |
| *S*-Indan-1-ol | 146 | 6.1 | 0.042 | 330 | 24 | 0.075 | 520 | 28 | 0.053 | 240[a] | 25 | 0.10[a] |
| Androsterone | 0.5 | 2.6 | 5 2 | 1.0 | 17 | 17 | 1.2 | 20 | 16 | 1.7 | 18 | 11 |
| 5α-Androstane-3α,17β-diol | 0.8 | 5 2 | 6.5 | 1 6 | 24 | 15 | 2.1 | 28 | 13[a] | 1.0 | 19 | 19 |
| 5β-Pregnane-3α,20β-diol | 0.2 | 1 2 | 6 0 | 0 4 | 6.5 | 16[a] | 0.5[a] | 6.8 | 14[a] | 0 4[a] | 5 6 | 14[a] |
| Lithocholic acid | 1.0 | 1 9 | 1.9 | 0.6[a] | 2.8 | 4.6[a] | 0.6[a] | 3.0[a] | 5.0[a] | 0.5[a] | 2 6 | 5.2[a] |
| Pyridine-4-aldehyde[b] | 358 | 2.5 | 0 007 | 210 | 6.3 | 0.030 | 250 | 7 4 | 0 030 | 260 | 4 2[a] | 0.016[a] |
| 5α-Dihydrotestosterone[b] | 0.4 | 1 6 | 4 0 | 0.8[a] | 4.5 | 5.6[a] | 0.8 | 5 3 | 6 6 | 0.7[a] | 4.0 | 5.7[a] |

The values determined without activator are taken from Deyashiki *et al.* (*14*). [a]The standard deviations of the values range from 16 to 25%, although those of the other values are less than 15% [b]The reductase activity for the substrates was assayed.

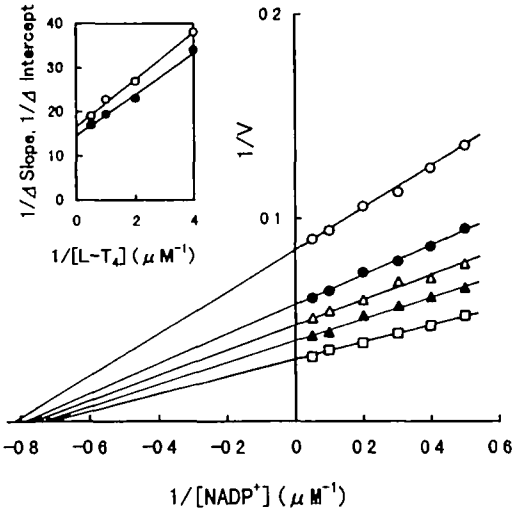



Fig. 3. **Effect of L-$T_4$ on the androsterone dehydrogenase activity as a function of $NADP^+$ concentration.** The activity was assayed with different concentrations of $NADP^+$ in the presence of a fixed concentration of 5 μM androsterone. L-$T_4$ concentrations: 0 μM (○), 0.25 μM (●), 0.5 μM (△), 1 0 μM (▲), and 2.0 μM (□) Initial velocity (*V*) is expressed as munits/ml. Replots of the 1/change in slope (●) and intercept (○) against 1/[activator] are shown in the inset.

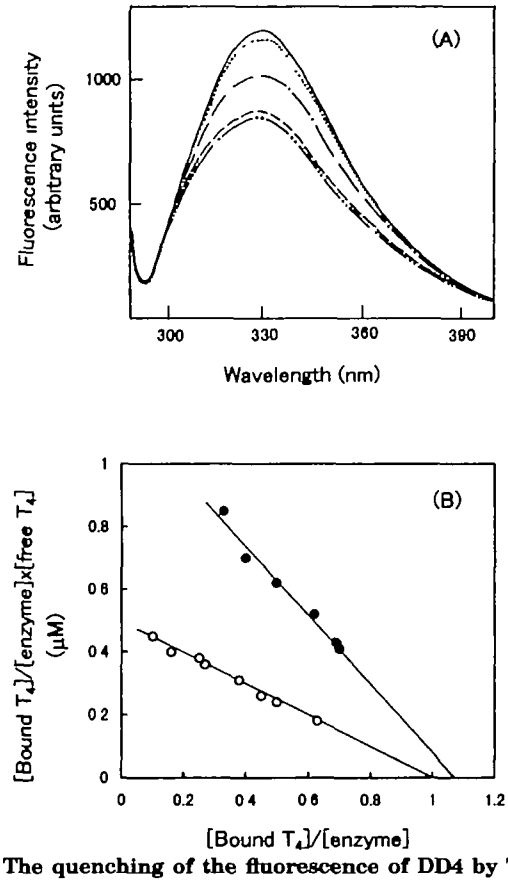



Fig. 4 **The quenching of the fluorescence of DD4 by $T_4$ and $rT_3$.** (A) 0 5 μM L-$T_4$ (——), 1 μM D-$T_4$ (— —), 1 μM DL-$rT_3$ (· · ·), or 1 μM L-$T_3$ (– –) was added to 1 μM DD4 solution in 0.1 M potassium phosphate, pH 7.4. The fluorescence of the enzyme solution without the compounds (—) was measured as the control. The excitation wavelength was 280 nm (B) Scatchard plot of the titration data of the enzyme with L-$T_4$ (●) or D-$T_4$ (○).

activated by the $T_4$ analogs exceeds that of the non-activated enzyme.

Since the $T_4$ analogs activate the activity of DD4 that occurs in their absence, the activation is a non-essential type of activation in which the binding constant ($K_A$) for an activator can be calculated from the initial velocity analysis with respect to substrate concentration in the presence of different fixed concentrations of activator (*21*). The dependency of the activity of DD4 on the $NADP^+$ concentration was analyzed at different fixed concentrations of L-$T_4$, because the reaction catalyzed by the enzyme follows an Ordered bi bi mechanism in which a coenzyme binds first to the enzyme (*14*). The lines of Lineweaver-Burk plots intersected below the 1/[$NADP^+$] axis, and replots of the reciprocal of change in the slope or intercept of the respective primary reciprocal plot data *versus* 1/[activator] were linear (Fig. 3). Similar results were obtained with D-$T_4$ or $rT_3$ as the activator. The results suggest that the kinetic pathway of activation follows the general non-essential activation system (Scheme 1, *21*), in which the activator binds to both the free enzyme and the enzyme-$NADP^+$ complex. The values of $K_A$, α and β (changes in $K_m$ for $NADP^+$ and $V_{max}$, respectively, by binding of the activator) for L-$T_4$ were 1.2 μM, 1.2, and 3.7, respectively, the respective values for D-$T_4$ were 1.5 μM, 1.1, and 3.2, and those for DL-$rT_3$ were 3.6 μM, 2.4, and 2.8. The $K_A$ values for L-$T_4$ and D-$T_4$ are lower than those of known drug activators (*16–18*), suggesting that at least L-$T_4$ and D-$T_4$ are good endogenous activators with high affinity for DD4.

Thyroxines have been reported to quench the fluorescence of tryptophan when they bind to proteins (*23*). The intrinsic fluorescence of DD4 was quenched by the addition of L-$T_4$ and D-$T_4$ (Fig. 4a), and then the enzyme was titrated

with L-$T_4$ and D-$T_4$ by monitoring the decrease in the fluorescence to confirm their high affinity for the enzyme. Typical titration data were graphed as Scatchard plots (Fig. 4b), and the plots derived from titrations with both L-$T_4$ and D-$T_4$ were linear and indicated that 1 mol $T_4$ binds per mol of monomeric enzyme. The dissociation constants ($K_d$) for L-$T_4$ and D-$T_4$ were calculated to be 0.8 and 1.0 μM, respectively, which are essentially identical to the $K_A$ values determined kinetically. DL-r$T_3$ also significantly quenched the intrinsic fluorescence of the enzyme, but a linear Scatchard-plot was not obtained, probably because of the usage of the racemic form. In contrast, the fluorescence quenching of the enzyme by L-$T_3$ was very low (Fig. 4a), and this hormone had no significant effect on the enzyme activity. This suggests a low affinity of L-$T_3$ for DD4, which again supports the structural importance of the iodo group at position 5′ for the binding of $T_4$ to the enzyme.

The kinetic activation mechanisms for $T_4$ and r$T_3$ are the same as those for the known drug activators, which have been reported to bind to an identical site on the enzyme based on combined activator experiments and attenuation of the activation for the mutant enzymes K270M and R276M (*16–18*). The proposed activation mechanism by the drug activators is that, in addition to a conformational change, the interaction of their sulfonyl or carboxyl groups with Lys-270 and/or Arg-276 weakens the binding of the coenzyme and results in an increase in turnover. To test whether the binding site for the drug activators is identical to that for the $T_4$ analogs, the combined effects of the drugs and $T_4$ analogs on the stimulatory effect of D-$T_4$ were first examined (Fig. 5). The stimulation percentages of the two activators were not additive when they were mixed, and the mixed activators instead lowered the stimulatory effect caused by high concentrations of D-$T_4$. Similarly, mixing its

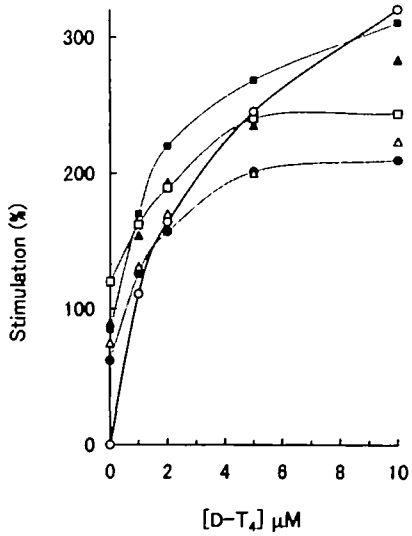



Fig. 5. **Effects of combined activators on the stimulatory effect by D-$T_4$.** The activity of DD4 was measured in the presence of different concentrations of D-$T_4$, as well as one of the combined stimulatory drugs (closed lines) or hormones (broken lines): 50 μM clofibric acid (■), 25 μM fenoprofen (□), 2.5 μM sulfobromophthalein (●), 1 μM L-$T_4$ (▲), and 5 μM DL-r$T_3$ (△). The control activity (○) was assayed in the absence of the combined activator. Values are expressed as the stimulation percentage [$(v-v_o)/v_o \times 100$], where $v$ and $v_o$ represent the velocities in the absence and presence, respectively, of D-$T_4$ and/or the combined activator.

enantiomer, L-$T_4$, also decreased the stimulation produced by high concentrations of D-$T_4$. This result suggests that the binding sites for the drug activators and the $T_4$ analogs are identical or overlapping, and that the activation efficiency of L- or D-r$T_3$ is higher than that of DL-r$T_3$ shown in Fig. 1. Second, the effects of L-$T_4$, D-$T_4$, and DL-r$T_3$ on the enzyme activity of the K270M and R276M mutant enzymes were examined to clarify whether the carboxyl group of the $T_4$ analogs interacts with the two basic residues of DD4. None of the $T_4$ analogs activated the enzyme activity of the mutant enzymes (Fig. 6). Thus, $T_4$ and r$T_3$ probably bind to the same site as the drug activators, and they may activate the enzyme activity through the same mechanism proposed for the drug activators.

Recently, the C-terminal loop of DD4 has been suggested to be involved in the binding of large drug activator molecules to the chimeric enzyme (CDD4-1) whose C-terminal 39 residues are replaced by those of DD1 (*20*). The replacement of the C-terminal sequence does not affect the stimulatory effect by small-size clofibric acid, whereas it decreases the stimulation by sulfobromophthalein, and abolishes that by bezafibrate. Since the influence of the anti-inflammatory 2-arylpropionic acids on CDD4-1 has not been studied, the effects of ibuprofen and fenoprofen, in addition to L-$T_4$, D-$T_4$, and r$T_3$, were examined. In contrast to other drug activators, the conversion of DD4 to CDD4-1 enhanced the stimulatory effects of the anti-inflammatory drugs (Fig. 6). This enhancement may be caused by preventing the binding of the drugs to the inhibitor site of DD4, because the drugs act as activators and inhibitors depending on their concentrations, and have been suggested to bind to a high-affinity activator site and/or low-affinity inhibitor site (*18*). The stimulatory effects of L-$T_4$, D-$T_4$, and r$T_3$ on CDD4-1 were low compared with those on the wild-type DD4. This change is similar to the case of sulfobromophthalein, but not to those observed for clofibric acid and the anti-inflammatory drugs. Since both sulfobromophthalein and $T_4$ (or r$T_3$) possess halogenated aromatic rings in their molecules, the hydrophobic aromatic ring or halogen group(s) on the ring may interact with the C-terminal domain of the enzyme.

*Inhibition Mechanism and Binding Site of Deamino Derivatives of $T_4$ and $T_3$*— $T_4$PA and $T_3$PA inhibited DD4 uncompetitively with respect to $NADP^+$ and competitively with respect to substrate, showing $K_i$ values of 42 and 60 nM, respectively (Fig. 7). The $K_i$ values are as low as those for such known potent DD4 inhibitors as phenolphthalein, medroxyprogesterone acetate and hexestrol (*14*, *20*). While these known inhibitors also inhibit human 3α-HSD isozymes other than DD4 (*6*, *10*, *20*), $T_4$PA specifically inhibited DD4 as described above. $T_4$PA would be useful as a specific inhibitor to discriminate DD4 from the other isozymes. When the effect of $T_4$PA on DD4 activated by D-$T_4$ was examined, its $IC_{50}$ (concentration required for 50% inhibition) value decreased significantly as the concentration of D-$T_4$ increased (Fig. 7). The increase in the inhibition potency of $T_4$PA by activation is not simply due to the increase in the $K_m$ and $V_{max}$ values for the substrate, because $T_4$PA also competitively inhibited the activated enzyme and the $K_i$ value decreased upon increasing the concentration of the activator. The kinetic results indicate that $T_4$PA binds to the enzyme-$NADP^+$ complex in either the presence or absence of the activator, *i.e.*, the enzyme proba-

bly has two distinct binding sites for the activator and inhibitor. Since D-$T_4$ binds to the free enzyme and enzyme-$NADP^+$ complex, the conformational change of the enzyme induced by the binding of the activator may increase the affinity for $T_4$PA.

To determine the inhibitor-binding site, the effects of $T_4$PA and $T_3$PA on the mutant and chimeric enzymes of DD4 were examined (Fig. 6). The inhibitory potency of $T_4$PA and $T_3$PA for K270M and R276M increased slightly compared with that for the wild-type enzyme, but the deamino derivatives did not show significant inhibition of CDD4-1, and, conversely, low activation was observed at low concentrations of $T_4$PA. The abolishment of the inhibition of $T_4$PA and $T_3$PA by the conversion of DD4 to CDD4-1 is similar to the cases of ibuprofen and fenoprofen, which act as inhibitors at high concentrations. A previous study on the inhibitory effects of the anti-inflammatory drugs (*18*) suggested that the carboxyl group of the drugs interacts with a specific anionic site delineated by C4N of $NADP^+$ and the side chains of Tyr and His at the active site of DD4, which has been shown by crystallographic studies of other AKR family proteins (*24–26*). Since $T_4$PA and $T_3$PA were competitive inhibitors and have a carboxyl group, this group of compounds may also bind to the anionic site at the active site of DD4. $T_4$, $T_3$, thyronine, and D-tyrosine with a carboxyl group did not inhibit CDD4-1 (data not shown), but have a positively charged amino group on their α-carbons that may prevent their access to the inhibitor site. The above data clearly indicate that the competitive inhibitors also interact with the residues in the C-terminal domain that have been shown to be responsible for the substrate-binding site of DD4 and other AKR family proteins (*20*, *25*, *26*). Considering that the carboxyl groups of $T_4$PA and $T_3$PA bind to the anionic site at the active site in the substrate-binding cleft, their hydrophobic iodinated aromatic ring(s) may interact with residues in the C-terminal domain of DD4. The low activation of this chimeric enzyme by $T_4$PA but not $T_3$PA can be explained as follows. The conformational change in the C-terminal domain induced by the conversion of DD4 to the chimeric enzyme may weaken the binding of the inhibitory compounds to the anionic site or to other parts in the substrate-binding site. In addition, the conformational change might allow the binding of $T_4$PA to the activator site of CDD4-1, because $T_4$PA but not $T_3$PA has an iodo group at position 5′, which is one of the structural requisites for stimulation by $T_4$ analogs.

The proposed binding sites for the inhibitory and stimulatory $T_4$ analogs seem to overlap with respect to the interactions of their hydrophobic aromatic rings with the C-terminal domains of DD4. However, the kinetic data indi-

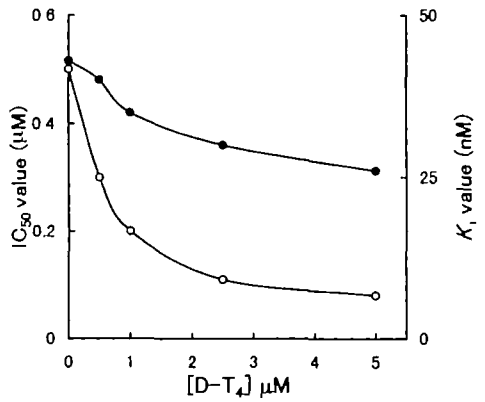



Fig. 7 **Effect of D-$T_4$ on the inhibitory potency of $T_4$PA.** The $IC_{50}$ (○) and $K_i$ (●) values for $T_4$PA were determined in the presence of different concentrations of D-$T_4$, for which the inhibition patterns were all competitive with respect to *S*-indan-1-ol.

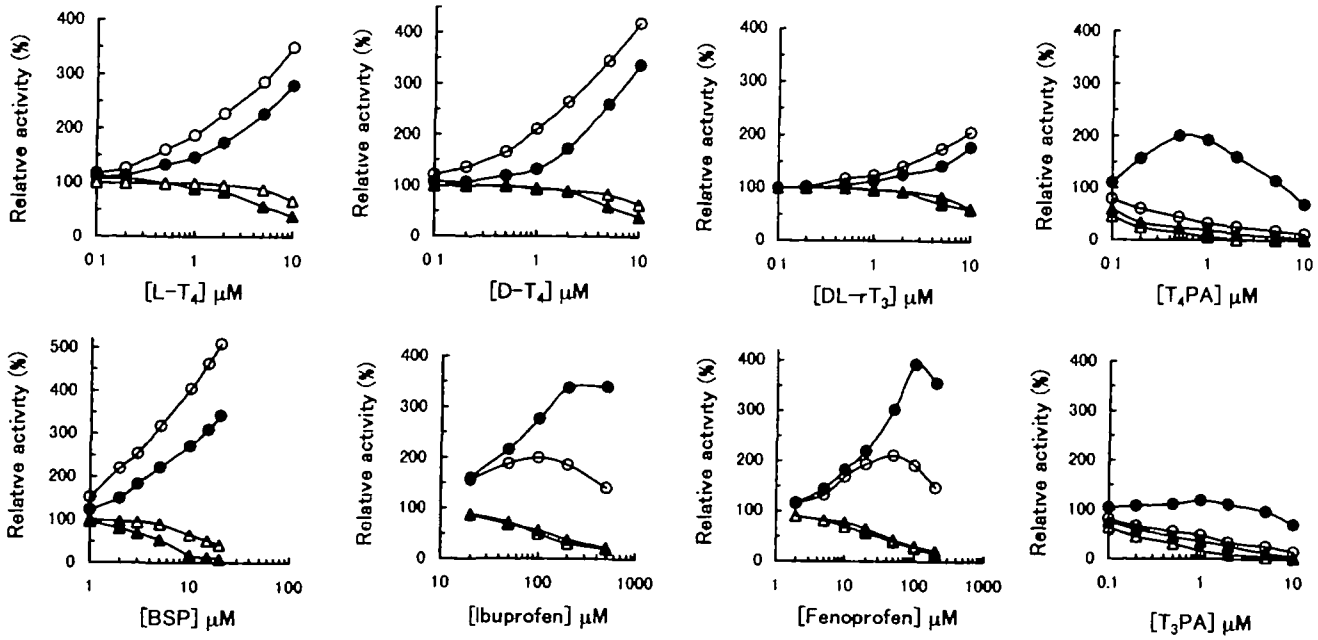



Fig. 6. **Effects of fenoprofen, ibuprofen, and $T_4$ derivatives on the dehydrogenase activities of the mutant and chimeric enzymes.** The activities of the wild-type DD4 (○), K270M (▲), R276M (△), and CDD4-1 (●) were assayed in the absence and presence of L-$T_4$, D-$T_4$, DL-$rT_3$, fenoprofen, ibuprofen, $T_4$PA, and $T_3$PA, and are expressed relative to the control activities of the respective enzymes determined without the drugs and $T_4$ derivatives. The data on the effect of sulfobromophthalein (BSP) are taken from Matsuura *et al.* (*20*).

cate the simultaneous binding of both inhibitor and activator to the enzyme, and also suggest differences in the interaction sites of their carboxyl groups, *i.e.*, the orientation of this group in the inhibitor is near the C4N of the nicotinamide ring of the coenzyme, whereas that of the activator is near the 2′-phosphate of the adenine ribose moiety. In addition, the influence of the conversion of DD4 to CDD4-1 on the inhibitory effects of $T_4$PA and $T_3$PA was much greater than that on the stimulatory effects of $T_4$ and $rT_3$. Therefore, the C-terminal residues responsible for the binding of the inhibitory and stimulatory $T_4$ analogs are probably different, although direct binding studies are needed.

*Physiological Significance of the Binding of $T_4$ Analogs*— The results presented in this study show that DD4 binds L-$T_4$, D-$T_4$, and $rT_3$, but not L-$T_3$, which exhibits the highest biological activity of the thyroid hormones. There are reports in the literature that several $NAD^+$-dependent dehydrogenases, such as glutamate dehydrogenase (*27*), malate dehydrogenase (*28*), glycerol-3-phosphate dehydrogenase (*29*), alcohol dehydrogenase (*30*), and aldehyde dehydrogenase (*31*), are inhibited by $T_4$ and/or $T_3$. DD4 is the first dehydrogenase that is not only $NADP^+$-dependent but also activated by the binding of $T_4$ and $rT_3$. The affinity of DD4 for these compounds is lower by one order of magnitude than the $K_i$ values of human alcohol and aldehyde dehydrogenases, which have been suggested to be modulated by thyroid hormones (*30*) and to act as weak binding proteins for thyroid hormones (*31*). A number of high-affinity thyroid-hormone-binding proteins have been identified in the cytosol of various tissues (*32–38*). Most of the high-affinity binding proteins have $K_d$ values for $T_3$ in the nanomolar range, but their affinities for $T_4$ and $rT_3$ are low (*35–38*). Although the $K_A$ and $K_d$ values of DD4 for $T_4$ and $rT_3$ are in the micromolar range, the binding specificity of DD4 for thyroid hormones is in contrast to that of the high-affinity and specific thyroid-hormone-binding proteins. At elevated intracellular concentrations of thyroid hormones in human liver, DD4 would act as a binding protein for the less-active thyroid hormones that modulate the enzyme activity.

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